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(71) Applicant: **IDEC PHARMACEUTICALS CORPORATION** [US/US]; 3030 Callan Road, San Diego, CA 92121 (US).

(72) Inventor: **HANNA, Nabil**; 14770 Avenida Insurgentes, Rancho Sante Fe, CA 92067 (US).

(74) Agents: **TESKIN, Robin, L.** et al.; Pillsbury Winthrop LLP, 1600 Tysons Boulevard, McLean, VA 22102 (US).

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(54) Title: COMBINATION THERAPY FOR TREATMENT OF AUTOIMMUNE DISEASES USING B CELL DEPLETING/IMMUNOREGULATORY ANTIBODY COMBINATION

(57) Abstract: The present invention concerns treatment of autoimmune diseases with the combination of an immunoregulatory antibody, e.g. and anti-B7.1 or anti-B7.2 or anti-CD40L antibody and at least one B cell antibody, such as CD19, CD20, CD22, CD23, or CD37, wherein such antibodies may be administered separately, or in combination, and in either, over prolonged periods of time.

Combination Therapy for Treatment of Autoimmune Diseases Using B Cell Depleting/ Immunoregulatory Antibody Combination

Field of the Invention

The present invention provides a novel combination therapy for treatment of autoimmune diseases. Particularly, the invention relates to the combined usage of an immunoregulatory antibody, preferably an antibody that modulates T and/or B cell differentiation, proliferation and/or function and a B cell depleting antibody for autoimmune disease therapy. These antibodies may be administered separately or in combination, and in either order.

Background of the Invention

Recently, the use of antibodies for treatment of diseases including cancers, especially non-Hodgkin's lymphoma, leukemias, viral mediated diseases, and autoimmune diseases has gained wide acceptance. In particular, the use of anti-CD20 or anti-CD22 antibodies that possess cell depleting activity for treatment of cancers, e.g., non-Hodgkin's lymphoma and related B cell lymphomas has been reported. Also, the use of B cell depleting antibodies specific to CD19 and CD37 has been reported, among others.

Further, the use of various immunoregulatory antibodies, i.e., antibodies that elicit a therapeutic benefit by modulating, i.e., enhancing or inhibiting a particular immune pathway has been reported. For example, such antibodies modulate the differentiation, proliferation, activation, and/or function of T or B cells, or other cells involved in regulation of immunity. Such immunoregulatory antibodies bind a ligand

or receptor on an immune cell, typically a B or T cell antigen that is involved in regulation of humoral or cellular immunity. Examples of such ligands are immune signaling molecules such as B7.1, B7.2, T cell regulatory molecules such as CD40-L, CD40, and CD4. A discussion of the function of some of these antigens and the prior use of antibodies specific thereto for therapy is briefly discussed below.

CD40L is a receptor expressed on the surface of activation of helper cells, and is the counterreceptor for CD40, a ligand expressed on the surface of B-lymphocytes as well as other antigen-presenting cells. The contact-dependent interaction of CD40L on activated T cells with CD40 expressed on B and other antigen-presenting cells, termed "T cell helper function" results in the activation and differentiation of B lymphocytes and is instrumental in the regulation of humoral immune responses. This regulation involves modulation of the specificity, secretion and isotype-encoded functions of antibody molecules. The process by which T cells help B cells to differentiate has been divided into two distinctive phases: the induction and effector phases (Vitetta *et al., Adv.Immunol.* 45:1 (1989); Noelle *et al., Immunol. Today* 11:361 (1990)).

The molecular basis for T cell help in humoral immunity via the interaction of CD40 and its ligand gp39 (also known as CD40L and CD154) is now well understood. Essentially, it is known that activated T helper cells express lymphokine genes and CD40L, a membrane protein that is essential for the reciprocal activation of cognate, antigen-presenting B cells. The interaction of CD40L with its receptor CD40 on a B cell, drives B cell entry and induces B cell responsiveness to the growth and differentiation effects of lymphokines.

Additionally, it is known that CD40L plays a larger, more general role in T cell immune processes, i.e., that is apart from its role in T cell help and the regulation of humoral immunity. This role of CD40L is not as well understood. For example, it is theorized that the pathology of T cell mediated autoimmune diseases including by way of example multiple sclerosis, type 1 diabetes, inflammatory bowel disease, oophoritis, and thyroiditis involves the presence of a particular CD40 ligand expressing T-suppressor cell population that play a role in disease pathology, perhaps by overriding the role of T effector cells. Also, the role of CD40 and CD40L in

peripheral and central to tolerance and its contribution to autoimmune disease has been reported. (Durie *et al., Res. Immunol. Vol* 145(3):200-205 (1994)).

The use of antagonists of CD40L for the treatment of both B cell-mediated and T cell-mediated autoimmune diseases has been reported. For example, EP 555,880, U.S. Patent 5,474,771, and WO 93/09212 disclose the use of CD40L antagonists for treating humoral autoimmune disease. The use of CD40L antagonists to treat T cell mediated autoimmune diseases is disclosed in U.S. Patent 5833987, and its PCT counterpart PCT/US96/09B7.

As discussed, the use of molecules that specifically bind target antigens on B lymphocytes and which deplete B cells as therapeutic agents has also been reported. Probably the most well accepted B cell target for therapy is the CD20 antigen, given the FDA's approval of Rituxan®, a chimeric monoclonal antibody directed against the CD20 antigen for treatment of non-Hodgkin's lymphoma.

The CD20 antigen (also called human B-lymphocyte-restricted differentiation antigen, -p-33) is a hydrophobic transmembrane protein with a molecular-weight of approximately 35 kD located on pre-B and mature B lymphocytes (Valentine *et al. J. Biol. Chem.* 264(19):11282-11287 (1989); and Einfeld *et al. EMBO J.* 7(3):711-717 (1988)). The antigen is also expressed on greater than 90% of B cell non-Hodgkin's lymphomas HL Anderson *et al. Blood* 63(6): 1424-1433 (1984)), but is not found on hematopoietic stem cells, pro-B cells, normal plasma cells or other normal tissues (Tedder *et al. J. Immunol.* 135(2):973-979 (1985)). CD20 regulates early steps) in the activation process for cell cycle initiation and differentiation (Tedder *et al., supra)* and possibly functions as a calcium ion channel (Tedder *et al. J. Cell. Biochem.* 14D:195 (1990)).

Given the expression of CD20 in B cell lymphomas, this antigen can serve as a candidate for "targeting" of such lymphomas. In essence, such targeting can be generalized as follows: antibodies specific to the CD20 surface antigen of B cells are administered to a patient; these anti-CD20 antibodies specifically bind to the CD20 antigen of (ostensibly) both normal and malignant B cells; and the antibodies bound to the CD20 surface antigen leads to the destruction and depletion of neoplastic B cells. Additionally, chemical agents or radioactive labels having the potential to destroy the tumor can be conjugated to the anti-CD20 antibody such that the agent is

specifically "delivered" to the neoplastic B cells. Irrespective of the approach, a primary goal is to destroy the tumor; the specific approach can be determined by the particular anti-CD20 antibody which is utilized and, thus, the available approaches to targeting the CD20 antigen can vary considerably.

CD19 is another antigen that is expressed on the surface of cells of the B lineage. Like CD20, CD19 is found on cells throughout differentiation of the lineage from the stem cell stage up to a point just prior to terminal differentiation into plasma cells (Nadler, L. *Lymphocyte Typing* II2:3-37 and Appendix, Renling *et al.* eds. (1986) by Springer Verlag). Unlike CD20, antibody binding to CD19 causes internalization of the CD19 antigen. CD19 antigen is identified by the HD237-CD19 antibody (also called the "B4" antibody) (Kiesel et al. *Leukemia Research II,* 12:1119 (1987)), among others. The CD19 antigen is present on 4-8% of peripheral blood mononuclear cells and on greater than 90% of B cells isolated from peripheral blood, spleen, lymph node or tonsil. CD19 is not detected on peripheral blood T cells, monocytes or granulocytes. Virtually all non T cell acute lymphoblastic leukemias (ALL), B cell chronic lymphocytic leukemias (CLL) and B cell lymphomas express CD19 detectable by the antibody B4 (Nadler *et al. J. Immunol.* 131:244 (1983); and Nadler *et al. in Progress in Hematology Vol. XII pp.*187-206. Brown, E. ed. (1981) by Grune & Stratton, Inc.

CD22 is another antigen that is expressed on the surface of cells of the B lineage. This antigen is also referred to by the names "BL-CAM" and "LyB8". This antigen is a membrane immunoglobulin-associated protein having a molecular weight of about 140,000, that is tyrosine-phosophorylated when membrane Ig is ligated thereto. (Engel *et al. J. R&PMed* 181(4):1521-1526 91995); Campbell and Eur. *J.Immunol.* 25:1573). This antigen has been reported to be a negative regulator of B-cell receptor signaling (Nitschke, *et al., Curr. Biol.* 7:133 (1997); and to promote monocyte erythrocyte athism (Stemenkoul *et al. Nature* 345:74 (1990)). A naked antibody specific to CD22, referred to as Lymphocide™ is now in clinical trials for the treatment of indolent non-Hodgkin's lymphoma by Immunomedics, Inc. Also, the use of an yttrium 90 labeled form of this same antibody for treating indolent and aggressive non-Hodgkin's lymphomas is also in clinical trials.

CD23 is still another antigen expressed on B cells and is the low affinity receptor for IgE, also known as FcERII. The use of antibodies that bind CD23 for treatment of inflammatory, autoimmune and allergic disorders has been suggested in the patent and non-patent literature.

B7.1 and B7.2 comprise other examples of B cell antigens to which the use of ligands that specifically bind, and which act as immunoregulators has been reported to possess therapeutic utility. Particularly, it has been reported that anti-B7, particularly those that bind to B7. 1 (CD80), B7.2 (CD86), or B7.3 transmembrane glycoproteins expressed on the surface of B cells have potential application as immunosuppressants and for treatment of various diseases. For example, U.S. Patent 5,869,040 issued February 9, 1999 to DeBoer et al., and assigned to Chiron Corporation discloses the use of anti-B7.1 antibodies in combination with another immunosuppressant for treating transplant rejection, graft-vs-host disease and rheumatoid arthritis. Also, U.S. Patent 5,885,579, issued March 23, 1999 to Linsley et al., discloses the treatment of immune diseases involving T cell interactions with B7 positive cells by the administration of a ligand specific for a B7 antigen, e.g. B7.1(CD80) or B7.2 (CD86).

Further, U.S. Patent 6,113,198 to Anderson et al. discloses the use of antibodies specific for B7-1 antigen which, in contrast to previous anti-B7 antibodies, do not inhibit the B7.1/CTLA-4 interaction and are useful for the treatment of diseases including autoimmune diseases. However, the combined usage of these antibodies with an antibody specific to CD40L is not disclosed, nor is the use of the antibody in conjunction with a B cell depleting antibody reported.

The Rituximab (RITUXAN®) antibody, in particular, is a genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen. RITUXAN® is indicated for the treatment of patients with relapsed or refractory low grade or follicular, CD20 positive, B cell non-Hodgkin's lymphoma (U.S. Patent No. 5,736,B7 issued April 7, 1998 to Anderson et al.). In vitro mechanism of action studies have demonstrated that RITUXAN® binds human complement and lyses lymphoid B cell lines through complement- dependent cytotoxicity (CDC) (Reff *et al. Blood* 83(2):435 445 (1994)). Additionally, it has significant activity in assays for antibody-dependent cellular cytotoxicity (ADCC). More recently, RITUXAN® has been shown to have anti-proliferative effects in

tritiated thymidine incorporation assays and to induce apoptosis directly, while other anti-CD 19 and CD20 antibodies do not (Maloney *et al. Blood* 88(10):637a (1996)). Synergy between RITUXAN® and chemotherapies and toxins has also been observed experimentally. In particular, RITUXAN® sensitizes drug-resistant human B cell lymphoma cell lines to the cytotoxic effects of doxorubicin, CDDP, VP-16, diphtheria toxin and ricin (Demidem *et al. Cancer Chemotherapy & Radiopharmaceuticals* 12(3):177-186 (1997)). *In vivo* RITUXAN® very effectively depletes B cells from the peripheral blood, lymph nodes, and bone marrow of cynomolgus monkeys, presumably through complement and cell-mediated processes (Reff *et al. Blood* 83(2):435-445 (1994)).

Perrotta and Abuel *Blood:92 Abstract* #3360 from ASH 40th Annual Meeting (November, 1998) provides an anecdotal report of a fifty-year old female with idiopathic thrombocytopenic purpura (ITP) responding to RITUXAN®.

Summary of the Invention

The invention is directed to the treatment of an autoimmune disease, preferably a B cell mediated autoimmune disease, using the combination of at least one immunoregulatory antibody and at least one B cell depleting antibody, e.g., an antibody that targets CD20, CD19, CD22, CD23, or CD37. Administration of these types of antibodies separately or in combination elicits a synergistic benefit when used for treating autoimmune diseases. This results because the B cell depleting antibody functions to deplete B cell numbers and therefore reduce the amount of circulating IgE and other antibodies that are involved in the pathology of autoimmunity. However, the B cell depleting antibody, e.g. RITUXAN®, tends to preferentially deplete activated B cells. By contrast, immunoregulatory antibodies, e.g., anti-B7 and anti-CD40L antibodies elicit their immunoregulatory effect, i.e., immunosuppression on non-activated B cells, i.e., non-activated antigen presenting B cells. Therefore, the use of these two functionally distinct types of antibodies is hypothesized to elicit a synergistic benefit in that it facilitates the removal of both activated and non-activated B cells from the circulation. Thereby, circulating levels of autoimmune antibodies will significantly decrease because the levels of antibody-producing B cells will decrease dramatically. This will afford significant therapeutic

benefits, especially in autoimmune diseases wherein B cells, and more particularly autoantibodies elicit an active involvement in disease pathology.

As discussed below, preferred immunoregulatory antibodies include anti-B7.1 or anti-B7.2, anti-CD40, anti-CD40L, and anti-CD4 antibodies. Preferred examples of B cell depleting antibodies include those specific to CD20, CD19, CD21, CD37 and CD22.

In one aspect of the present invention there is provided use of an immunoregulatory antibody and an antibody having a B cell depleting activity for the manufacture of a combined preparation for simultaneous, separate or sequential use in treating an autoimmune disease in a mammal wherein the immunoregulatory antibody is selected from anti-CD80(B7.1), anti-CD86(B7.2), anti-CD40 and anti-CD4 antibody.

In a second aspect of the present invention there is provided an article of manufacture comprising a container and one or several compositions contained therein, wherein at least one composition comprises a B cell depleting antibody, and at least another composition comprises an anti-CD80 (B7.1) or anti-CD86 (B7.2) antibody and further comprising a package insert instructing the user of the composition to treat a patient having or predisposed to an autoimmune disease.

In a third aspect of the present invention there is provided use of an antibody to CD80 (B7.1) or CD86 (B7.2) and an anti-CD20 antibody having substantial B cell depleting activity, for the manufacture of a combined preparation for simultaneous, separate or sequential use in treating a disease selected from the group consisting of multiple sclerosis, ITP, lupus, diabetes, rheumatoid arthritis, psoriasis, thyroiditis, dermatitis, and IBD.

In one embodiment there is provided a combination therapy for treatment of an autoimmune disease, e.g. rheumatoid arthritis, SLE, ITP, by the combined usage of (i) an immunoregulatory antibody, preferably one that inhibits non-activated B cells; and (ii) a B cell depleting antibody; wherein such antibodies may be administered separately or in combination, and in either order.

In a more specific embodiment there is provided the treatment of an autoimmune disease by the combined usage of (i) an antibody to B7.1 or B7.2 and/or anti-CD40L, and (ii) a B cell depleting antibody selected from an anti-CD20, anti-CD19, anti-CD22 and anti-CD37.

The invention further pertains to articles of manufacture for treatment of autoimmune diseases which comprise a container and one or more compositions contained therein, which comprise an effective amount of an immunoregulatory antibody, e.g. anti-CD40L or anti-B7.1 or anti-B7.2 antibody (immunoregulatory antibody) and a B cell depleting antibody or fragment thereof, anti-CD20, anti-CD19, anti-CD22 or anti-CD37 (B cell depleting antibody).

Detailed Description of the Preferred Embodiments

I. Definitions

"B Cell Depleting Antibody" therein is an antibody or fragment that binds to a B cell marker which upon administration, results in demonstrable B cell depletion. Preferably, such antibody, after administration, typically within about several days or less, will result in a depletion of B cell number by about 50% or more. In a preferred embodiment, the B cell depleting antibody will be RITUXAN® (a chimeric anti-CD20 antibody) or one having substantially the same or greater cell depleting activity. This antibody has been demonstrated to provide substantially 90% of B cell depletion within 24 hours of administration in an effective amount.

"Immunoregulatory Antibody" refers to an antibody that elicits an effect on the immune system by a mechanism different from depletion of activated B cells. Examples thereof include antibodies that inhibit T cell immunity, B cell immunity, e.g. by inducing tolerance (anti-CD40L, anti-CD40) or other immunosuppressant antibodies (anti-B7.1, anti-B7.2, or anti-CD4). In some instances, the immunoregulatory antibody of immune cells may also possess the ability to potientiate apoptosis.

A "B cell surface marker" herein is an antigen expressed on the surface of a B cell which can be targeted with an antagonist which binds thereto. Exemplary B cell surface markers include the CD10, CD19, CD20, CD21, CD22, CD23, CD24, CD37, CD53, CD72, CD73, CD74, CDw75, CDw76, CD77, CDw78, CD79a, CD79b, CD80 (B7.1), CD81, CD82, CD83, CDw84, CD85 and CD86 (B7.2) leukocyte surface markers. The B cell surface marker of particular interest is preferentially expressed on B cells compared to other non-B cell tissues of a mammal and may be expressed on both precursor B cells and mature B cells. In one embodiment, the marker is one, like CD20 or CD 19, which is found on B cells throughout differentiation of the lineage from the stem cell stage up to a point just prior to terminal differentiation into plasma cells. The preferred B cell surface markers herein are CD 19, CD20, CD23, CD80 and CD86.

The "CD20" antigen is a ~35 kDa, non-glycosylated phosphoprotein found on the surface of greater than 90% of B cells from peripheral blood or lymphoid organs. CD20 is expressed during early pre-B cell development and remains until plasma cell differentiation. CD20 is present on both normal B cells as well as malignant B cells. Other names for CD20 in the literature include "B-lymphocyte- restricted antigen" and "Bp35". The CD20 antigen is described in Clark *et al. PNAS (USA)* 82:1766(1985).

The "CD19" antigen refers to a ~90kDa antigen identified, for example, by the HD237-CD19 or B4 antibody (Kiesel *et al. Leukemia Research II,* 12: 1119 (1987)). Like CD20, CD19 is found on cells throughout differentiation of the lineage from the stem cell stage up to a point just prior to terminal differentiation into plasma cells. Binding of an antagonist to CD19 may cause internalization of the CD19 antigen.

The "CD22" antigen refers to an antigen expressed on B cells, also known as "BL-CAM" and "LybB" that is involved in B cell signaling and an adhesion. (See Nitschke *et al., Curr. Biol.* 7:133 (1997); Stamenkovic *et al., Nature* 345:74 (1990)). This antigen is a membrane immunoglobulin-associated antigen that is tyrosine phosphorylated when membrane Ig is ligated. (Engel *et al., J. Etyp. Med.* 181(4):1521, 1586 (1995)). The gene encoding this antigen has been cloned, and its Ig domains characterized.

B7 antigen includes the B7.1 (CD80), B7.2 (CD86) and B7.3 antigen, which are transmembrane antigens expressed on B cells. Antibodies which specifically bind B7 antigens, including human B7.1 and B7.2 antigens are known in the art. Preferred B7 antibodies comprise the primatized® B7 antibodies disclosed by Anderson et al. in U.S. Patent No. 6,113,198, assigned to IDEC Pharmaceuticals Corporation, as well as human and humanized B7 antibodies.

CD23 refers to the low affinity receptor for IgE expressed by B and other cells. In the present invention, CD23 will preferably be human CD23 antigen. CD23 antibodies are also known in the art. Most preferably, in the present invention, the CD23 antibody will be a human or chimeric anti-human CD23 antibody comprising human IgGI or IgG3 constant domains, and most preferably the depleting anti-CD23 antibodies disclosed in U.S. Patent No. 6,011,138.

An "autoimmune disease" is a non-malignant disease or disorder arising from and directed against an individual's own tissues. The non-malignant autoimmune diseases herein specifically exclude malignant or cancerous diseases or conditions, especially excluding B cell lymphoma, acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), Hairy cell leukemia and chronic myeloblastic leukemia. Examples of such diseases or disorders include inflammatory responses such as inflammatory skin diseases including psoriasis and dermatitis *(e.g.* atopic dermatitis); systemic scleroderma and sclerosis; responses associated with inflammatory bowel disease (such as Crohn's disease and ulcerative colitis); respiratory distress syndrome (including adult respiratory distress syndrome; ARDS); dermatitis; meningitis; encephalitis; uveitis; colitis; glomerulonephritis; allergic conditions such as eczema and asthma and other conditions involving infiltration of T cells and chronic inflammatory responses; atherosclerosis; leukocyte adhesion

deficiency; rheumatoid arthritis; systemic lupus erythematosus (SLE); diabetes mellitus (*e.g.* Type I diabetes mellitus or insulin dependent diabetes mellitis); multiple sclerosis; Reynaud's syndrome; autoimmune thyroiditis; allergic encephalomyelitis; Sjorgen's syndrome; juvenile onset diabetes; and immune responses associated with acute and delayed hypersensitivity mediated by cytokines and T-lymphocytes typically found in tuberculosis, sarcoidosis, polymyositis, granulomatosis and vasculitis; pernicious anemia (Addison's disease); diseases involving leukocyte diapedesis; central nervous system (CNS) inflammatory disorder; multiple organ injury syndrome; hemolytic anemia (including cryoglobinemia); myasthenia gravis; antigen-antibody complex mediated diseases; anti-glomerular basement membrane disease; antiphospholipid syndrome; allergic neuritis; Graves' disease; Lambert-Eaton myasthenic syndrome; pemphigoid bullous; pemphigus; autoimmune polyendocrinopathies; Reiter's disease; stiff-man syndrome; Behcet disease; giant cell arteritis; immune complex nephritis; IgA nephropathy; IgM polyneuropathies; immune thrombocytopenic purpura (ITP), autoimmune thrombocytopenia and oophoritus.

A B cell "antagonist" is a molecule which, upon binding to a B cell surface marker, destroys or depletes B cells in a mammal and/or interferes with one or more B cell functions, *e.g.* by reducing or preventing a humoral response elicited by the B cell. By contrast, a B cell depleting antibody depletes B cells (*i.e.* reduce circulating B cell levels) in a mammal treated therewith. Such depletion may be achieved via various mechanisms such antibody-dependent cell-mediated cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC), inhibition of B cell proliferation and/or induction of B cell death (*e.g.* via apoptosis). Antagonists within the scope of the present invention include antibodies, synthetic or native sequence peptides and small molecule antagonists which bind to the B cell marker, optionally conjugated with or fused to a cytotoxic agent.

A CD40L antagonist is a molecule that specifically binds CD40L and preferably antagonizes the interaction of CD40L and CD40. Examples thereof include antibodies and antibody fragments that specifically bind CD40L, soluble CD40, soluble CD40 fusion proteins, and small molecules that bind CD40L. The preferred

antagonist according to the invention comprises an antibody or antibody fragment specific to CD40.

"Antibody-dependent cell-mediated cytotoxicity" and "ADCC" refer to a cell mediated reaction in which nonspecific cytotoxic cells that express Fc receptors (FcRs) (*e.g.* Natural Killer (NK) cells, neutrophils, and macrophages) recognize bound antibody on a target cell and subsequently cause lysis of the target cell. The primary cells for mediating ADCC, NK cells, express FcγRIII only, whereas monocytes express FcγRI, FcγRII and FcγRIII. FcR expression on hematopoietic cells in summarized is Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol.* 9:457-92 (1991). To assess ADCC activity of a molecule of interest, an *in vitro* ADCC assay, such as that described in US Patent No. 5,500,362 or 5,821,337 may be performed. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Alternatively, or additionally, ADCC activity of the molecule of interest may be *assessed in vivo, e.g.,* in a animal model such as that disclosed in Clynes *et al. PNAS (USA)* 95:652-656 (1998).

"Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. Preferably, the cells express at least FcγRIII and perform ADCC effector function. Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with PBMCs and NK cells being preferred. The effector cells may be isolated from a native source thereof, *e.g.* from blood or PBMCs as described herein.

The terms "Fc receptor" or "FcR" are used to describe a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the FcγRI, FcγRII, and FcγRII subclasses, including allelic variants and alternatively spliced forms of these receptors. FcγRII receptors include FcγRIIA (an "activating receptor") and FcγRUB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor FcγRIIA contains an immunoreceptor tyrosine-based

activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor FcγRIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (See review M. Daeon, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet, *Annu. Rev. Immunol.* 9:457-92 (1991); Capel et al., *Immunomethods* 4:25-34 (1994); and de Haas *et al., J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer *et al., J. Immunol.* 117:587 (1976) and Kim *et al., J. Immunol.* 24:249 (1994)).

"Complement dependent cytotoxicity" or "CDC" refers to the ability of a molecule to lyse a target in the presence of complement. The complement activation pathway is initiated by the binding of the first component of the complement system (Clq) to a molecule (e.g. an antibody) complexed with a cognate antigen. To assess complement activation, a CDC assay, e.g. as described in Gazzano-Santoro et al., *J. Immunol. Methods* 202:163 (1996), may be performed.

"Growth inhibitory" antagonists are those which prevent or reduce proliferation of a cell expressing an antigen to which the antagonist binds. For example, the antagonist may prevent or reduce proliferation of B cells in vitro and/or in vivo.

Antagonists which "induce apoptosis" are those which induce programmed cell death, e.g. of a B cell, as determined by binding of annexin V, fragmentation of DNA, cell shrinkage, dilation of endoplasmic reticulum, cell fragmentation, and/or formation of membrane vesicles (called apoptotic bodies).

The term "antibody" herein is used in the broadest sense and specifically covers intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of an intact antibody, preferably comprising the antigen-binding or variable region thereof. Examples of antibody fragments include Fab, Fab', $F(ab')^2$, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

"Native antibodies" are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (VH) followed by a number of constant domains. Each light chain has a variable domain at one end (VL) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light-chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light chain and heavy chain variable domains.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called hypervariable regions both in the light chain and the heavy chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FRs). The variable domains of native heavy and light chains each comprise four FRs, largely adopting a 13-sheet configuration, connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the B -sheet structure. The hypervariable regions in each chain are held together in close proximity by the FRs and, with the hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat *et al., Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody dependent cellular cytotoxicity (ADCC).

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin

treatment yields an F(ab')2 fragment that has two antigen-binding sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and antigen-binding site. This region consists of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association. It is in this configuration that the three hypervariable regions of each variable domain interact to define an antigen-binding site on the surface of the VH-VL dimer. Collectively, the six hypervariable regions confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (CHI) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CHI domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear at least one free thiol group. F(ab')Z antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa and lambda, based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy chains, antibodies can be assigned to different classes. There are five major classes of intact antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgGI, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of antibodies are called alpha, delta, epsilon, gamma and mu, respectively. Preferably, the heavy-chain constant domains will complete the gamma-1, gamma-2, gamma-3 and gamma-4 constant region. Preferably, these constant domains will also comprise modifications to enhance antibody stability such as the P and E modification disclosed in U.S. Patent No. 6,011,138 incorporated by reference in its entirety herein. The

subunit structures and three dimensional configurations of different classes of immunoglobulins are well known.

"Single-chain Fv" or "scFv" antibody fragments comprise the VH and VL domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the Fv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see Pluckthun in *The Pharmacology of Monoclonal Antibodies, vol.* 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (VH) connected to a light chain variable domain (VL) in the same polypeptide chain (VH - VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger *et al., Proc. Natl. Acad.* Sci. USA, 90:6444-6448 (1993).

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.,* the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al., Nature,* 256:495

(1975), or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson *et al., Nature*, 352:624-628 (1991) and Marks *et al., J. Mol. Biol.*, 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chains is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; Morrison *et al., Proc. Natl. Acad. Sci. USA,* 81:6851-6855 (1984)). Chimeric antibodies of interest herein include "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate *(e.g.* Old World Monkey, Ape, etc.) and human constant region sequences.

"Humanized" forms of non-human *(e.g.*, murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human

immunoglobulin. For further details, see Jones *et al., Nature* 32 1:522-525 (1986); Riechmann *et al., Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (*e.g.* residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat *et al., Sequences of Proteins of Immunological Interest,* 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991)), and/or those residues from a "hypervariable loop" (*e.g.* residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain; Chothia and Lesk. *J. Mol. Biol.* 196:901-917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

An antagonist "which binds" an antigen of interest, *e.g.* a B cell surface marker, is one capable of binding that antigen with sufficient affinity such that the antagonist is useful as a therapeutic agent for targeting a cell, i.e. a B cell, expressing the antigen.

An "anti-CD20 antibody" herein is an antibody that specifically binds CD20 antigen, preferably human CD20, having measurable B cell depleting activity, preferably having at least about 10% the B cell depleting activity of RITUXAN® (see U.S. Patent No. 5,736,137, incorporated by reference herein in its entirety), when administered in the same amount and conditions as RITUXAN®.

An "anti-CD22 antibody" herein is an antibody that specifically binds CD22 antigen, preferably human CD22, having measurable B cell depleting activity, preferably having at least about 10% the B cell depleting activity of RITUXAN® (see U.S. Patent No. 5,736,137, incorporated by reference herein in its entirety), when administered in the same amount and conditions as RITUXAN®.

An "anti-CD19 antibody" herein is an antibody that specifically binds CD19 antigen, preferably human CD19, having measurable B cell depleting activity,

preferably having at least about 10% the B cell depleting activity of RITUXAN® (see U.S. Patent No. 5,736,137, incorporated by reference herein in its entirety), when administered in the same amount and conditions as RITUXAN®.

An "anti-CD23 antibody" herein is an antibody that specifically binds CD23 antigen, preferably human CD23, having measurable B cell depleting activity, preferably having at least about 10% the B cell depleting activity of RITUXAN® (see U.S. Patent No. 5,736,137, incorporated by reference herein in its entirety), when administered in the same amount and conditions as RITUXAN®.

An "anti-CD37 antibody" herein is an antibody that specifically binds CD37 antigen, preferably human CD37, having measurable B cell depleting activity, preferably having at least about 10% the B cell depleting activity of RITUXAN® (see U.S. Patent No. 5,736,137, incorporated by reference herein in its entirety), when administered in the same amount and conditions as RITUXAN®.

An "anti-B7 antibody" herein is an antibody that specifically binds B7.1, B7.2 or B7.3, most preferably human B7.1. Preferably this antibody will specifically inhibit B7/CD28 interactions and more preferably inhibit B7.1/CD28 interactions and will not substantially inhibit B7/CTLA-4 interactions. Even more preferably, the anti-B7.1 antibody will be one of the specific antibodies described in U.S. Patent 6,113,898, incorporated by reference in its entirety herein.

An "anti-CD40L antibody" is an antibody that specifically binds CD40L (also known as CD154, gp39, TBAM), preferably one having agonistic activity. A preferred anti-CD40L antibody is one having the specificity of a humanized antibody disclosed in U.S. Patent No. 6,011,358 (assigned to IDEC Pharmaceuticals Corporation), incorporated by reference in its entirety herein.

An "anti-CD4 antibody" is one that specifically binds CD4, preferably human CD4, more preferably a primatized or humanized anti-CD4 antibody, preferably a human gamma 4 anti-human CD4 antibody.

An "anti-CD40 antibody" is an antibody that specifically binds CD40, preferably human CD40, such as those disclosed in U.S. Patent 5,874,085, 5,874,082, 5,801,227, 5,674,442, and 5,667,165, all of which are incorporated by reference herein.

Preferably, both the B cell depleting antibody and the immunoregulatory antibody will contain human constant domains. Suitable antibodies may include IgG1, IgG2, IgG3 and IgG4 isotypes.

Specific examples of antibodies which bind to CD20 antigen include: "rituximab" ("RITUXAN®") (US Patent No. 5,736,137, expressly incorporated herein by reference); yttrium-[90]-labeled 2B8 murine antibody "Y2B8" (US Patent No. 5,736,137, expressly incorporated herein by reference); murine IgG2a "B1" optionally labeled with 131I antibody (BEXXAR™) (U.S. Patent No. 5,595,721, expressly incorporated herein by reference); murine monoclonal antibody "1F5" (Press *et al. Blood* 69(2):584-591 (1987); and "chimeric 2H7" antibody (U.S. Patent No. 5,677,180, expressly incorporated herein by reference).

Specific examples of antibodies which bind CD22 include Lymphocide™ reported by Immunomedics, now in clinical trials for non-Hodgkin's lymphoma. Examples of antibodies that bind B7 antigen include the B7 antibody reported U.S. Patent No. 5,885,577, issued to Linsley et al., the anti-B7 antibody reported in U.S. Patent No. 5,869,050, issued to DeBoer et al., and assigned to Chiron Corporation, and the primatized® anti-B7.1 (CD80) antibody disclosed in U.S. Patent No. 6,113,198 to Anderson et al., all of which are incorporated by reference in their entirety.

Preferred examples of antibodies that bind CD23 include the primatized® antibodies specific to human CD23 reported by Reff *et al.,* in U.S. Patent 6,011,138, issued on July 4, 1999, co-assigned to IDEC Pharmaceuticals Corp. and Seikakagu Corporation of Japan. Other anti-CD23 antibodies and antibody fragments include those reported by Bonnefoy *et al.,* No. 96 12741; Rector *et al. J. Immunol.* 55:481-488 (1985); Flores-Rumeo *et al. Science* 241:1038-1046 (1993); Sherr *et al. J. Immunol.,* 142:481-489 (1989); and Pene *et al., PNAS, USA* 85:6820-6824 (1988). Such antibodies are reportedly useful for treatment of allergy, autoimmune diseases, and inflammatory diseases.

The terms "rituximab" or "RITUXAN®" herein refer to the genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen and designated "C2B8" in US Patent No. 5,736,B7, expressly incorporated herein by reference. The antibody is an IgGI kappa immunoglobulin containing

murine light and heavy chain variable region sequences and human constant region sequences. Rituximab has a binding affinity for the CD20 antigen of approximately 8.OnM.

An "isolated" antagonist is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the antagonist, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antagonist will be purified (1) to greater than 95% by eight of antagonist as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated antagonist includes the antagonist *in situ* within recombinant cells since at least one component of the antagonist's natural environment will not be present. Ordinarily, however, isolated antagonist will be prepared by at least one purification step.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disease or disorder as well as those in which the disease or disorder is to be prevented. Hence, the mammal may have been diagnosed as having the disease or disorder or may be predisposed or susceptible to the disease.

The expression "therapeutically effective amount" refers to an amount of the antagonist which is effective for preventing, ameliorating or treating the autoimmune disease in question.

The term "immunosuppressive agent" as used herein for adjunct therapy refers to substances that act to suppress or mask the immune system of the mammal being treated herein. This would include substances that suppress cytokine production, downregulate or suppress self-antigen expression, or mask the MHC antigens.

Examples of such agents include 2-amino-6-aryl-5-substituted pyrimidines (see U.S. Pat. No. 4,665,077, the disclosure of which is incorporated herein by reference), azathioprine; cyclophosphamide; bromocryptine; danazol; dapsone; glutaraldehyde (which masks the MHC antigens, as described in U.S. Pat. No. 4,120,649); anti-idiotypic antibodies for MHC antigens and MHC fragments; cyclosporin A; steroids such as glucocorticosteroids, *e.g.*, prednisone, methylprednisolone, and dexamethasone; cytokine or cytokine receptor antagonists including anti-interferon-α, β- or δ-antibodies, anti-tumor necrosis factor-α antibodies, anti-tumor necrosis factor-β antibodies, anti-interleukin-2 antibodies and anti-IL-2 receptor antibodies; anti-LFA-1 antibodies, including anti-CD 1 la and anti-CD 18 antibodies; anti-L3T4 antibodies; heterologous anti-lymphocyte globulin; pan-T antibodies, preferably anti-CD3 or anti-CD4/CD4a antibodies; soluble peptide containing a LFA-3 binding domain (WO 90/08187 published 7/26/90), streptolanase; TGF-β; streptodornase; RNA or DNA from the host; FK506; RS-61443; deoxyspergualin; rapamycin; T-cell receptor (Cohen *et al.,* U.S. Patent No. 5,114,721); T-cell receptor fragments (Offner *et al., Science,* 251: 430-432 (1991); WO 90/11294; laneway, *Nature,* 341: 482 (1989); and WO 91/01133); and T cell receptor antibodies (EP 340,109) such as T10B9.

The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. At211 1131 1125 Y9o Re 186 Re lg8 sM153 Bi212 p32 and radioactive isotopes of Lu), chemotherapeutic agents, and toxins such as small molecule toxins or enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include alkylating agents such as thiotepa and cyclosphosphamide (CYTOXAN™); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphaoramide and trimethylolomelamime nitrogen mustards such as chiorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine

oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabicin, carminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK®; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2, 2',2"-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, e.g. paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, NJ) and doxetaxel (Taxotere, Rhone-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; esperamicins; capecitabine; and pharmaceutically acceptable

salts, acids or derivatives of any of the above. Also included in this definition are anti-hormonal agents that act to regulate or inhibit hormone action on tumors such as anti-estrogens including for example tamoxifen, raloxifene, aromatase inhibiting 4(5)-imidazoles, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (Fareston); and antiandrogens such as flutamide, nilutamide, bicalutamide, leuprolide, and goserelin; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

The term "cytokine" is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor-α and -β; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); nerve growth factors such as NGF-13; platelet-growth factor; transforming growth factors (TGFs) such as TGF-α and TGF-β; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon-α, -β, and -γ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocytemacrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-la, IL-2, IL-g, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12, IL-15; a tumor necrosis factor such as TNF-α or TNF-β; and other polypeptide factors including LIF and kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines.

The term "prodrug" as used in this application refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the parent drug and is capable of being enzymatically activated or

converted into the more active parent form. See, *e.g.*, Wilman, "Prodrugs in Cancer Chemotherapy," *Biochemical Society Transactions,* 14, pp. 375-382, 615th Meeting Belfast (1986) and Stella *et al.*, "Prodrugs: A Chemical Approach to Targeted Drug Delivery," *Directed Drug Delivery,* Borchardt *et al.*, (ed.), pp. 247-267, Humana Press (1985). The prodrugs of this invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate-containing prodrugs, peptide-containing prodrugs, D-amino acid-modified prodrugs, glycosylated prodrugs,13-lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs which can be converted into the more active cytotoxic free drug. Examples of cytotoxic drugs that can be derivatized into a prodrug form for use in this invention include, but are not limited to, those chemotherapeutic agents described above.

A "liposome" is a small vesicle composed of various types of lipids, phospholipids and/or surfactant which is useful for delivery of a drug (such as the antagonists disclosed herein and, optionally, a chemotherapeutic agent) to a mammal. The components of the liposome are commonly arranged in a bilayer formation, similar to the lipid arrangement of biological membranes.

The term "package insert" is used to refer to instructions customarily included in commercial packages of therapeutic products, that contain information about the indications, usage, dosage, administration, contraindications and/or warnings concerning the use of such therapeutic products.

II. Production of Antibodies

The methods and articles of manufacture of the present invention use, or incorporate, an antibody that has immunoregulatory activity, e.g. anti-B7, anti-CD40L or anti-CD40, and an antibody that binds to a B cell surface marker having B depleting activity. Accordingly, methods for generating such antibodies will be described herein.

The molecule to be used for production of, or screening for, antigen(s) may be, *e.g.*, a soluble form of the antigen or a portion thereof, containing the desired epitope. Alternatively, or additionally, cells expressing the antigen at their cell surface can be used to generate, or screen for, antagonist(s). Other forms of the B cell surface

marker useful for generating antagonists will be apparent to those skilled in the art. Suitable antigen sources for CD40L, CD40, CD19, CD20, CD22, CD23, CD37 and B7 (B7.1 or B7.2) antigen for producing antibodies according to the invention are well known.

Preferably, the CD40L antibody or anti-CD40L antibody will be the humanized anti-CD40L antibody disclosed in U.S. Patent 6,001,358, issued on June 14, 1999, and assigned to IDEC Pharmaceuticals Corporation.

While a preferred CD40L antagonist is an antibody, antagonists other than antibodies are contemplated herein. For example, the antagonist may comprise soluble CD40, a CD40 fusion protein or a small molecule antagonist optionally fused to, or conjugated with, a cytotoxic agent (such as those described herein). Libraries of small molecules may be screened against the B cell surface marker of interest herein in order to identify a small molecule which binds to that antigen. The small molecule may further be screened for its antagonistic properties and/or conjugated with a cytotoxic agent.

The antagonist may also be a peptide generated by rational design or by phage display (W098/35036 published 13 August 1998), for example. In one embodiment, the molecule of choice may be a "CDR mimic" or antibody analogue designed based on the CDRs of an antibody, for example. While the peptide may be antagonistic by itself, the peptide may optionally be fused to a cytotoxic agent or to an immunoglobulin Fc region *(e.g.,* so as to confer ADCC and/or CDC activity on the peptide).

A description follows as to exemplary techniques for the production of the antibody antagonists used in accordance with the present invention.

(i) Polyclonal antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.,* keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine

residues), glutaraldehyde, succiic anhydride, SOC l_2, or $R^1N=C=NR$, where R and R^I are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, *e.g.* 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to fourteen days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(ii) Monoclonal antibodies

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, *i.e.,* the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al., Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as herein above described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro.* Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice,* pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma

cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Manassas, Virginia, USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.,* 133:300 1 (1984); Brodeur *et al., Monoclonal Antibody Production Techniques and Applications,* pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the 30 Scatchard analysis of Munson *et al., Anal. Biochem.,* 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice,* pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPML-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin

purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra *et al., Curr. Opinion in Immunol.*, 5:256-262 (1993) and Pluckthun, *Immunol. Revs.*, 130:151-188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty *et al., Nature,* 348:552-554(1990). Clackson *et al., Nature,* 352:624-628 (1991) and Marks *et al., J. Mol. Biol.,* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks *et al., Bio/Technology,* 10:779-783 (1992)), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse *et al., Nuc. Acids. Res.*, 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4,816,567; Morrison, *et al., Proc. Natl Acad. ScL USA,* 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically, such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of

one antigencombining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iii) Humanized antibodies

Methods for humanizing non-human antibodies have been described in the art. Preferably, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al., Nature,* 321:522-525 (1986); Riechmann *et al., Nature,* 332:323-327 (1988); Verhoeyen *et al., Science,* 239:1534-1536 (1988)), by substituting hypervariable region sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some hypervariable region residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework region (FR) for the humanized antibody (Suns *et al., J. Immunol.,* 151:2296 (1993); Chothia *et al., J. Mol. Biol.,* 196:901 (1987)). Another method uses a particular framework region derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter *et al., Proc. Natl. Acad. Sci. USA,* 89:4285 (1992); Presta *et al., J. Immunol.,* 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this

goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three- dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, especially the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the hypervariable region residues are directly and most substantially involved in influencing antigen binding.

(iv) Human antibodies

As an alternative to humanization, human antibodies can be generated. For example, it is now possible to produce transgenic animals *(e.g.,* mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region PH) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ line mutant mice will result in the production of human antibodies upon antigen challenge. See, *e.g.,* Jakobovits *et al., Proc. Mad. Acad. Sci. USA,* 90:255 1 (1993); Jakobovits *et al., Nature,* 362:255-258 (1993); Bruggermann *et al., Year in immuno.,* 7:33 (1993); and U.S. Patent Nos. 5,591,669, 5,589,369 and 5,545,807.

Alternatively, phage display technology (McCafferty *et al., Nature* 348:552-553 (1990)) can be used to produce human antibodies and antibody fragments *in vitro,* from immunoglobulin variable (V) domain gene repertoires from non-immunized donors. According to this technique, antibody V domain genes are cloned in-frame into either a major or minor coat protein gene of a filamentous

bacteriophage, such as M13 or fd, and displayed as functional antibody fragments on the surface of the phage particle. Because the filamentous particle contains a single-stranded DNA copy of the phage genome, selections based on the functional properties of the antibody also result in selection of the gene encoding the antibody exhibiting those properties. Thus, the phage mimics some of the properties of the B cell. Phage display can be performed in a variety of formats; for their review see, *e.g.,* Johnson, Kevin S. and Chiswell, David J., *Current Opinion in Structural Biology* 3:564-57 1 (1993). Several sources of V-gene segments can be used for phage display. Clackson *et al., Nature,* 352:624-628 (1991) isolated a diverse array of anti-oxazolone antibodies from a small random combinatorial library of V genes derived from the spleens of immunized mice. A repertoire of V genes from unimmunized human donors can be constructed and antibodies to a diverse array of antigens (including self antigens) can be isolated essentially following the techniques described by Marks *et al., J. Mol. Biol,* 222:581-597 (1991), or Griffith *et al., EMBO J.* 12:725-734 (1993). See also, U.S. Patent Nos. 5,565,332 and 5,573,905.

Human antibodies may also be generated by in *vitro* activated B cells (see U.S. Patent Nos. 5,567,610 and 5,229,275).

(v) Antibody fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, *e.g.,* Morimoto *et al., Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan *et al., Science,* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form F(ab')2 fragments (Carter *et al., Bio/Technology* 10: 163-167 (1992)). According to another approach, F(ab')2 fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv). See WO 93/16185; U.S. Patent No. 5,571,894; and U.S. Patent No. 5,587,458. The antibody fragment may also be a "linear antibody", *e.g.,* as described in U.S. Patent No.

5,641,870, for example. Such linear antibody fragments may be monospecific or bispecific.

(vi) Bispecific antibodies

Bispecific antibodies are antibodies that have binding specificities for at least two different epitopes. Exemplary bispecific antibodies may bind to two different epitopes of the B cell surface marker. Other such antibodies may bind a first B cell marker and further bind a second B cell surface marker. Alternatively, an anti-B cell marker binding arm may be combined with an arm which binds to a triggering molecule on a leukocyte such as a T cell receptor molecule (*e.g.* CD2 or CD3), or Fc receptors for IgG (FcR), such as FcRI (CD64), FcRII (CD32) and FcRIII (CD16) so as to focus cellular defense mechanisms to the B cell. Bispecific antibodies may also be used to localize cytotoxic agents to the B cell. These antibodies possess a B cell marker-binding arm and an arm which binds the cytotoxic agent (*e.g.* saporin, anti-interferon-α, vinca alkaloid, ricin A chain, methotrexate or radioactive isotope hapten). Bispecific antibodies can be prepared as full length antibodies or antibody fragments (*e.g.* F(ab)2 bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein *et al., Nature,* 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker *et al., EMBO J.,* 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CHI) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs

encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh *et al., Methods in Enzymology,* 121:210 (1986).

According to another approach described in U.S. Patent No. 5,731,168, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the CH3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (*e.g.* tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chains) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (*e.g.* alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the

other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan *et al., Science,* 229:81(1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate F(ab')2 fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby *et al., J. Exp. Med.,* 175:2 17-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling *in vitro* to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny *et al., J. Immunol.* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form

monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger *et al., Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al., J. Immunol.,* 152:5368 (1994).

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt *et al. J. Immunol.* 147: 60(1991).

III. Conjugates and Other Modifications of the Antagonist

The antagonist used in the methods or included in the articles of manufacture herein is optionally conjugated to a cytotoxic agent.

Chemotherapeutic agents useful in the generation of such antagonist-cytotoxic agent conjugates have been described above.

Conjugates of an antagonist and one or more small molecule toxins, such as a calicheamicin, a maytansine (U.S. Patent No. 5,208,020), a trichothene, and CC 1065 are also contemplated herein. In one preferred embodiment of the invention, the antagonist is conjugated to one or more maytansine molecules (e.g. about 1 to about 10 maytansine molecules per antagonist molecule). Maytansine may, for example, be converted to May SS-Me which may be reduced to May-SH3 and reacted with modified antagonist (Charm *et al. Cancer Research* 52:127-131(1992)) to generate a maytansinoid-antagonist conjugate.

Alternatively, the antagonist is conjugated to one or more calicheamicin molecules. The calicheamicin family of antibiotics are capable of producing double stranded DNA breaks at sub-picomolar concentrations. Structural analogues of calicheamicin which may be used include, but are not limited to, γ_1^I, α_2^I, α_3^I, N-acetyl-γ_1^I, PSAG and O^I_1 (Hinman *et al. Cancer Research* 53:3336-3342 (1993) and Lode *et al, Cancer Research* 58: 2925-2928 (1998)).

Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa),* ricin A chain, abrin A chain, modeccin A chain, alpha sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. See, for example, WO 93/21232 published October 28, 1993.

The present invention further contemplates antagonist conjugated with a compound with nucleolytic activity *(e.g.* a ribonuclease or a DNA endonuclease such as a deoxyribonuclease; DNase).

A variety of radioactive isotopes are available for the production of radioconjugated antagonists. Examples include At^{211}, I^{131}, I^{125}, Y^{90}, Re^{186}, RE^{188}, Sm^{153}, Bi^{212}, P^{32} and radioactive isotopes of Lu.

Conjugates of the antagonist and cytotoxic agent may be made using a variety of bifunctional protein coupling agents such as N-succinimidyl- 3-(2-pyriyldithiol) propionate (SPDP), succinimidyl-4-(N-maleimidomethyl) cyclohexane-l-carboxylate, iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2, 4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al. Science* 238: 1098 (1987). Carbon-14-labeled 1 isothiocyanatobenzyl-3- methyldiethylene triaminepentaacetic.acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antagonist. See W094/11026. The linker may be a "cleavable linker" facilitating release of the cytotoxic drug in the cell. For example, an acid-labile linker, peptidase-sensitive linker, dimethyl linker or disulfide-containing linker (Charm *et al. Cancer Research* 52:127-131 (1992)) may be used.

Alternatively, a fusion protein comprising the antagonist and cytotoxic agent may be made, *e.g.* by recombinant techniques or peptide synthesis.

In yet another embodiment, the antagonist may be conjugated to a "receptor" (such streptavidin) for utilization in tumor pretargeting wherein the antagonist-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent and then administration of a "ligand" *(e.g.* avidin) which is conjugated to a cytotoxic agent *(e.g.* a radionucleotide).

The antagonists of the present invention may also be conjugated with a prodrug activating enzyme which converts a prodrug *(e.g.* a peptidyl chemotherapeutic agent, see W081/01145) to an active anti-cancer drug. See, for example, WO 88/07378 and U.S. Patent No. 4,975,278.

The enzyme component of such conjugates includes any enzyme capable of acting on a prodrug in such a way so as to covert it into its more active, cytotoxic form.

Enzymes that are useful in the method of this invention include, but are not limited to, alkaline phosphatase useful for converting phosphate-containing prodrugs into free drugs; arylsulfatase useful for converting sulfate-containing prodrugs into free drugs; cytosine deaminase useful for converting non-toxic5-fluorocytosine into the anti-cancer drug, fluorouracil; proteases, such as serratia protease, thermolysin, subtilisin, carboxypeptidases and cathepsins (such as cathepsins B and L), that are useful for converting peptide-containing prodrugs into free drugs; D-alanylcarboxypeptidases, useful for converting prodrugs that contain D-amino acid substituents; carbohydrate cleaving enzymes such as 13-galactosidase and neuraminidase useful for converting glycosylated prodrugs into free drugs; 13-lactamase useful for converting drugs derivatized with 13-lactams into free drugs; and penicillin amidases, such as penicillin V amidase or penicillin G amidase, useful for converting drugs derivatized at their amine nitrogens with phenoxyacetyl or phenylacetyl groups, respectively, into free drugs. Alternatively, antibodies with enzymatic activity, also known in the art as "abzymes", can be used to convert the prodrugs of the invention into free active drugs (see, *e.g.,* Massey, *Nature* 328:457-458 (1987)). Antagonist-abzyme conjugates can be prepared as described herein for delivery of the abzyme to a tumor cell population.

Enzymes can be covalently bound to the antagonist by techniques well known in the art such as the use of the heterobifunctional crosslinking reagents discussed above. Alternatively, fusion proteins comprising at least the antigen binding region of an antagonist of the invention linked to at least a functionally active portion of an enzyme of the invention can be constructed using recombinant DNA techniques well known in the art (see, *e.g.,* Neuberger *et al., Nature,* 312:604-608 (1984)).

Other modifications of the antagonist are contemplated herein. For example, the antagonist may be linked to one of a variety of nonproteinaceous polymers, *e.g.,* polyethylene glycol, polypropylene glycol, polyoxyalkylenes, or copolymers of polyethylene glycol and polypropylene glycol.

The antibodies disclosed herein may also be formulated as liposomes. Liposomes containing the antagonist are prepared by methods known in the art, such as described in Epstein *et al., Proc. Natl. Acad. Sci. USA,* 82:3688 (1985); Hwang *et al., Proc. Natl Acad. Sci. USA,* 77:4030 (1980); U.S. Pat. Nos. 4,485,045 and 4,544,545; and W097/38731 published October 23, 1997. Liposomes with enhanced circulation time are disclosed in U.S. Patent No. 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of an antibody of the present invention can be conjugated to the liposomes as described in Martin *et al. J. Biol. Chem.* 257:286-288 (1982) via a disulfide interchange reaction. A chemotherapeutic agent is optionally contained within the liposome. See Gabizon *et al. J.National Cancer Inst.* 81(19)1484 (1989).

Amino acid sequence modification(s) of protein or peptide antagonists described herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antagonist. Amino acid sequence variants of the antagonist are prepared by introducing appropriate nucleotide changes into the antagonist nucleic acid, or by peptide synthesis. Such modifications include, for example, deletions from, and/or insertions into and/or substitutions of, residues within the amino acid sequences of the antagonist. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided

that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the antagonist, such as changing the number or position of glycosylation sites.

A useful method for identification of certain residues or regions of the antagonist that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells *Science,* 244:1081-1085 (1989). Here, a residue or group of target residues are identified (*e.g.,* charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with antigen. Those amino acid locations demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at, or for, the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to analyze the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed antagonist variants are screened for the desired activity.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Examples of terminal insertions include an antagonist with an N-terminal methionyl residue or the antagonist fused to a cytotoxic polypeptide. Other insertional variants of the antagonist molecule include the fusion to the N- or C-terminus of the antagonist of an enzyme, or a polypeptide which increases the serum half-life of the antagonist.

Another type of variant is an amino acid substitution variant. These variants have at least one amino acid residue in the antagonist molecule replaced by different residue. The sites of greatest interest for substitutional mutagenesis of antibody antagonists include the hypervariable regions, but FR alterations are also contemplated. Conservative substitutions are shown in Table 1 under the heading of "preferred substitutions." If such substitutions result in a change in biological activity, then more substantial changes, denominated "exemplary substitutions" in

Table 1, or as further described below in reference to amino acid classes, may be introduced and the products screened.

Table 1

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	val
Arg (R)	lys; gin; asn	lys
Asn (N)	gln; his; asp, lys; arg	gln
Asp (D)	glu; asn	glu
Cys (C)	ser; ala	ser
Gln (Q	asn; glu	asn
Glu (E)	asp; gin	asp
Gly (G)	ala	ala
His (H)	asn; gin; lys; arg	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; g1n; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala; tyr	tyr
Pro (P)	ala	ala
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr; phe	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

Substantial modifications in the biological properties of the antagonist are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

(2) neutral hydrophiuic: cys, ser, thr;

(3) acidic: asp, glu;

(4) basic: asn, gln, his, lys, arg;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another class.

Any cysteine residue not involved in maintaining the proper conformation of the antagonist also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking. Conversely, cysteine bonds) may be added to the antagonist to improve its stability (particularly where the antagonist is an antibody fragment such as an Fv fragment).

A particularly preferred type of substitutional variant involves substituting one or more hypervariable region residues of a parent antibody (*e.g.* a humanized or human antibody). Generally, the resulting variants selected for further development will have improved biological properties relative to the parent antibody from which they are generated. A convenient way for generating such substitutional variants is affinity maturation using phage display. Briefly, several hypervariable region sites (*e.g.* 6-7 sites) are mutated to generate all possible amino substitutions at each site. The antibody variants thus generated are displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. The phage-displayed variants are then screened for their biological activity (*e.g.* binding affinity) as herein disclosed. In order to identify candidate hypervariable region sites for modification, alanine scanning mutagenesis can be performed to identified hypervariable region residues contributing significantly to antigen binding. Alternatively, or in addition, it may be beneficial to analyze a crystal structure of the antigen-antibody complex to identify contact points between the antibody and antigen. Such contact residues and neighboring residues are candidates for substitution according to the techniques elaborated herein. Once such variants are generated, the panel of variants is subjected to screening as described herein and antibodies with superior properties in one or more relevant assays may be selected for further development.

Another type of amino acid variant of the antagonist alters the original glycosylation pattern of the antagonist. By altering is meant deleting one or more

carbohydrate moieties found in the antagonist, and/or adding one or more glycosylation sites that are not present in the antagonist.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly seine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the antagonist is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more seine or threonine residues to the sequence of the original antagonist (for O-linked glycosylation sites).

Nucleic acid molecules encoding amino acid sequence variants of the antagonist are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the antagonist.

It may be desirable to modify the antibodies used in the invention to improve effector function, e.g. so as to enhance antigen-dependent cell-mediated cyotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC) of the antagonist. This may be achieved by introducing one or more amino acid substitutions in an Fc region of an antibody antagonist. Alternatively or additionally, cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement- mediated cell killing and

antibody-dependent cellular cytotoxicity (ADCC). See Caron *et al., J. Exp Med.* 176:1191-1195 (1992) and Shopes, *B. J. Immunol.* 148:2918-2922 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff *et al. Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson *et al. Anti-Cancer Drug Design* 3:2 19-230 (1989).

To increase the serum half life of the antagonist, one may incorporate a salvage receptor binding epitope into the antagonist (especially an antibody fragment) as described in U.S. Patent 5,739,277, for example. As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (*e.g.,* IgGI, IgG2, IgG3, or IgG4) that is responsible for increasing the *in* vivo serum half-life of the IgG molecule.

IV. Pharmaceutical Formulations

Therapeutic formulations comprising antagonists used in accordance with the present invention are prepared for storage by mixing an antagonist having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.* Zn-protein complexes); and/or

non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

The immunoregulatory antibody or antibody fragment and the B cell depleting antibody antagonist may be in the same formulation or in different formulations. Administration can be concurrent or sequential, and is effected in either order. Such administration may be effected by repeated administration of both antibodies, for a prolonged period of time.

Exemplary anti-CD20 antibody formulations are described in W098/56418, expressly incorporated herein by reference. This publication describes a liquid multidose formulation comprising 40 mg/mL rituximab, 25 mM acetate, 150 mM trehalose, 0.9% benzyl alcohol, 0.02% polysorbate 20 at pH 5.0 that has a minimum shelf life of two years storage at 2-8°C. Another anti-CD20 formulation of interest comprises 10mg/mL rituximab in 9.0 mg/mL sodium chloride, 7.35 mg/mL sodium citrate dihydrate, 0.7mg/niL polysorbate 80, and Sterile Water for Injection, pH 6.5.

Lyophilized formulations adapted for subcutaneous administration are described in W097/04801. Such lyophilized formulations may be reconstituted with a suitable dilutent to a high protein concentration and the reconstituted formulation may be administered subcutaneously to the mammal to be treated herein.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. For example, it may be desirable to further provide a chemotherapeutic agent, cytokine or immunosuppressive agent (*e.g.* one which acts on T cells, such as cyclosporin or an antibody that binds T cells, *e.g.* one which binds LFA-1). The effective amount of such other agents depends on the amount of antagonist present in the formulation, the type of disease or disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as used hereinbefore or about from 1 to 99% of the heretofore employed dosages.

The active ingredients may also be entrapped in microcapsules prepared, for example, by 30 coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly- (methylmethacylate) microcapsules, respectively, in colloidal drug delivery systems (for example,

liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

Sustained-release preparations may be prepared. Suitable examples of sustained release preparations include semipermeable matrices of solid hydrophobic polymers containing the antagonist, which matrices are in the form of shaped articles, *e.g.* films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl- methacrylate), or poly(vinylalcohol)), polylactides (U.S. Patent No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, noir degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

V. Treatment with the B Cell Depleting Antibody and Immunoregulatory Antibody

One or more compositions comprising a B cell depleting antibody and/or an immunoregulatory antibody will be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular autoimmune disease or disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disease or disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The therapeutically effective amount of the antagonist to be administered will be governed by such considerations.

As noted previously, the B cell depleting antibody and the immunoregulatory antibody may be in the same or in different formulations. These antibody formulations can be administered separately or concurrently, and in either order. Preferably, the B cell depleting antibody specific to the B cell antigen target, *e.g.*, CD20, CD19, CD22, CD23 or CD37, will be administered separately from the immunoregulatory antibody, *e.g.*, an anti-CD40L antibody or anti-CD40, anti-B7.1,

anti-B7.2 antibody. Preferably, the CD40L antibody will be the humanized anti-CD40L antibody disclosed in U.S. Patent No. 6,001,358. This antibody has been shown to have efficacy in treatment of both T and B cell autoimmune diseases, *e.g.*, multiple sclerosis and ITP. Also, unlike another humanized anti-CD40L antibody *(5c8)* reported by Biogen, this antibody is not known to cause adverse hematologic events.

As a general proposition, the therapeutically effective amount of an antibody administered parenterally per dose will typically be in the range of about 0.1 to 500 mg/kg of patient body weight per day, with the typical initial range of antagonist used being in the range of about 2 to 100 mg/kg.

The preferred B cell depleting antibody is RITUXAN®. Suitable dosage for such antibody is, for example, in the range from about 20mg/m2 to about 1000mg/m^2. The dosage of the antibody may be the same or different from that presently recommended for RITUXAN® for the treatment of non-Hodgkin's lymphoma. For example, one may administer to the patient one or more doses of substantially less than 375mg/m2 of the antibody, *e.g.* where the dose is in the range from about 20mg/m^2 to about 250mg/m^2, for example from about 50mg/m^2 to about 200mg/m^2.

Moreover, one may administer one or more initial doses of the antibody followed by one or more subsequent dose(s), wherein the mg/m^2 dose of the antibody in the subsequent doses exceeds the mg/m^2 dose of the antibody in the initial dose(s). For example, the initial dose may be in the range from about 20mg/m^2 to about 250mg/m^2 *(e.g.* from about 50mg/m^2 to about 200mg/m^2) and the subsequent dose may be in the range from about 250mg/m^2 to about 1000mg/m^2.

As noted above, however, these suggested amounts of both immunoregulatory antibodies are subject to a great deal of therapeutic discretion. The key factor in selecting an appropriate dose and scheduling is the result obtained, as indicated above. For example, relatively higher doses may be needed initially for the treatment of ongoing and acute diseases. To obtain the most efficacious results, depending on the autoimmune disease or disorder, the antagonist is administered as close to the first sign, diagnosis, appearance, or occurrence of the disease or disorder as possible or during remissions of the disease or disorder.

The antibodies are administered by any suitable means, including parenteral, subcutaneous, intraperitoneal, intrapulmonary, and intranasal, and, if desired for local immunosuppressive treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. In addition, the antibody may suitably be administered by pulse infusion, *e.g.*, with declining doses of the antibody. Preferably the dosing is given by injections, most preferably intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic.

One additionally may administer other compounds, such as chemotherapeutic agents, immunosuppressive agents and/or cytokines with the antibodies herein. The combined administration includes co-administration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order, wherein preferably there is a time period while both (or all) active agents simultaneously exert their biological activities.

Aside from administration of antibodies to the patient the present application contemplates administration of antibodies by gene therapy. Such administration of nucleic acid encoding the antibodies is encompassed by the expression "administering a therapeutically effective amount of an antagonist." See, for example, W096/07321 published March 14, 1996 concerning the use of gene therapy to generate intracellular antibodies.

There are two major approaches to getting the nucleic acid (optionally contained in a vector) into the patient's cells: in vivo and ex vivo. For in vivo delivery the nucleic acid is injected directly into the patient, usually at the site where the antagonist is required. For ex vivo treatment, the patient's cells are removed, the nucleic acid is introduced into these isolated cells and the modified cells are administered to the patient either directly or, for example, encapsulated within porous membranes which are implanted into the patient (see, *e.g.* U.S. Patent Nos. 4,892,538 and 5,283,187). There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells *in vitro,* or *in vivo* in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells *in vitro* include the use of liposomes, electroporation, microinjection, cell fusion,

DEAF-dextran, the calcium phosphate precipitation method, etc. A commonly used vector for ex vivo delivery of the gene is a retrovirus.

The currently preferred *in vivo* nucleic acid transfer techniques include transfection with viral vectors (such as adenovirus, Herpes simplex I virus, or adeno associated virus) and lipid-based systems (useful lipids for lipid-mediated transfer of the gene are DOTMA, DOPE and DC-Chol, for example). In some situations it is desirable to provide the nucleic acid source with an agent that targets the target cells, such as an antibody specific for a cell surface membrane protein or the target cell, a ligand for a receptor on the target cell, etc. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake, *e.g.* capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, and proteins that target intracellular localization and enhance intracellular half-life. The technique of receptor-mediated endocytosis is described, for example, by Wu *et al., J. Biol. Chem.* 262:4429-4432 (1987); and Wagner *et al., Proc. Natl. Acad. Sci. USA* 87:3410-3414(1990). For review of the currently known gene marking and gene therapy protocols see Anderson *et al., Science* 256:808-813 (1992). See also WO 93/25673 and the references cited therein.

VI. Articles of Manufacture

In another embodiment of the invention, an article of manufacture containing materials useful for the treatment of the diseases or disorders described above is provided.

The article of manufacture comprises a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds or contains a composition which is effective for treating the disease or disorder of choice and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). As whole, there may be one or several compositions. At least one active agent in one of those compositions is an antibody having B cell depleting activity and at least one antibody is an immunoregulatory antibody such as an anti-CD40L, anti-CD40, anti-CD4 or anti-B7 antibody. The

label or package insert indicates that the composition is used for treating a patient having or predisposed to an autoimmune disease, such as those listed hereinabove. The article of manufacture may further comprise a second container comprising a pharmaceutically acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

Further details of the invention are illustrated by the following non-limiting Examples. The disclosures of all citations in the specification are expressly incorporated herein by reference.

Example 1

Patients with clinical diagnosis of rheumatoid arthritis (RA) are initially treated with rituximab (RITUXAN®) antibody. This patient may or may not also have a B cell depleting antibody, i.e., malignancy. Moreover, the patient is optionally further treated with any one or more agents employed for treating RA such as salicylate; nonsteroidal anti-inflammatory drugs such as indomethacin, phenylbutazone, phenylacetic acid derivatives *(e.g.* ibuprofen and fenoprofen), naphthalene acetic acids (naproxen), pyrrolealkanoic acid (tometin), indoleacetic acids (sulindac), halogenated anthranilic acid (meclofenamate sodium), piroxicam, zomepirac and diflunisal; antimalarials such as chloroquine; gold salts; penicillamine; or immunosuppressive agents such as methotrexate or corticosteroids in dosages known for such drugs or reduced dosages. Preferably however, the patient is only treated with RITUXAN®.

RITUXAN® is administered intravenously (IV) to the RA patient according to any of the following dosing schedules:

(A) 50Mg/M2 IV day 1
150 mg/m2 IV on days 8, 15 & 22

(B) 150Mg/M2 IV day 1
375 mg/m2 IV on days 8, 15 &22

(C) 375 Mg/M2 1V days 1, 8, 15 & 22

The patient is treated thereafter with a humanized anti-CD40L antibody disclosed in U.S. Patent No. 6,001,358 administered intravenously according to the same dosage regimen.

The primary response is determined by the Paulus index (Paulus *et al. Athritis Rheum.* 33:477-484 (1990)), *i.e.* improvement in morning stiffness, number of painful and inflamed joints, erythrocyte sedimentation (ESR), and at least a 2-point improvement on a 5-point scale of disease severity assessed by patient and by physician. Administration of RITUXAN® and the anti-CD40L antibody will alleviate one or more of the symptoms of RA in the patient treated as described above.

Example 2

Patients diagnosed with autoimmune hemolytic anemia (AIHA), e.g., cryoglobinemia or Coombs positive anemia, are treated with RITUXAN® antibody. AIHA is an acquired hemolytic anemia due to auto-antibodies that react with the patient's red blood cells. The patient treated optionally may also have a B cell malignancy. The patient is initially treated with a composition containing a humanized anti-human CD40L antibody, administered as a dosage of 500 mg/m2 given IV. This dosage is given twice a week for a total of four (4) weeks.

RITUXAN® is thereafter administered intravenously (IV) to the patient according to any of the following dosing schedules:

(A) 50Mg/M2 IV day 1
150mg/m2 IV on days 8,15 & 22

(B) 150Mg/M2 IV day 1
375mg/m2 IV on days 8,15 & 22

(C) 375mg/m2 IV days 1, 8, 15 & 22

Further adjunct therapies (such as glucocorticoids, prednisone, azathioprine, cyclophosphamide, vinca-laden platelets or Danazol) may be combined with the anti-CD40L antibody and RITUXAN® therapy. Preferably, the patient is treated with RITUXAN® and the same anti-CD40L antibody as in the previous example as the only other agent throughout the course of therapy.

Overall response rate is determined based upon an improvement in blood counts, decreased requirement for transfusions, improved hemoglobin levels and/or a decrease in the evidence of hemolysis as determined by standard chemical parameters.

Administration of the anti-CD40L antibody and RITUXAN® will improve any one or more of the symptoms of hemolytic anemia in the patient treated as described above.

Example 3

Adult immune thrombocytopenic purpura (ITP) is a relatively rare hematologic disorder that constitutes the most common of the immune-mediated cytopenias. The disease typically presents with severe thrombocytopenia that may be associated with acute hemorrhage in the presence of normal to increased megakaryocytes in the bone marrow. Most patients with ITP have an IgG antibody directed against target antigens on the outer surface of the platelet membrane, resulting in platelet sequestration in the spleen and accelerated reticuloendothelial destruction of platelets (Bussell, *J.B. Hematol. Oncol. Clin. North Am.* (4):179 (1990)). A number of therapeutic interventions have been shown to be effective in the treatment of ITP. Steroids are generally considered first-line therapy, after which most patients are candidates for intravenous immunoglobulin (IVIG), splenectomy, or other medical therapies including vincristine or immunosuppressive/cytotoxic agents. Up to 80% of patients with ITP initially respond to a course of steroids, but far fewer have complete and lasting remissions. Splenectomy has been recommended as standard second-line therapy for steroid failures, and leads to prolonged remission in nearly 60% of cases yet may result in reduced immunity to infection. Splenectomy is a major surgical procedure that may be associated with substantial morbidity (15%) and mortality (2%). IVIG has also been used as second line medical therapy, although only a small proportion of adult patients with ITP achieve remission.

Therapeutic options that would interfere with the production of autoantibodies by activated B cells without the associated morbidities that occur with corticosteroids and/or splenectomy would provide an important treatment approach for a proportion of patients with ITP.

Patients with clinical diagnosis of ITP (e.g. with a platelet count <75,000/μL) are treated with rituximab (RITUXAN®) antibody, optionally in combination with steroid therapy. The patient treated will not have a B cell malignancy.

RITUXAN® is again administered intravenously (IV) to the ITP patient according to any of the following dosing schedules:

(A) 50Mg/M2 IV day 1

150 mg/m2 IV on days 8, 15 & 22

(B) 150mg/m2 IV day 1

375mg/m2 IV on days 8, 15 & 22

(C) 375mg/m2 IV days 1, 8, 15 & 22

Concurrent with RITUXAN® administration, the patient is treated with one of the Primatized anti-B7.1 antibodies disclosed in U.S. Patent No. 6,113,898, incorporated in its entirety by reference herein. The anti-B7.1 antibody is administered intravenously in a separate formulation, at a dosage of 500 mg/m2, given twice a week for 3 weeks.

Patients are premedicated with one dose each of diphenhydramine 25-50 mg intravenously and acetaminophen 650 mg orally prior to the infusion of RITUXAN® and the anti-B7.1 antibody composition. Using a sterile syringe and a 21 gauge or larger needle, the necessary amount of RITUXAN® and anti-B7.1 antibody is transferred from the vial into an IV bag containing sterile, pyrogen-free 0.9% sodium chloride, USP (saline solution). The final concentration of RITUXAN® and B7.1 antibody is approximately 1 mg/mL. The initial dose infusion rate is initiated at 25 mg/hour for the first half hour then increased at 30 minute intervals by 50 mg/hr increments to a maximum rate of 200 mg/hours. If the first course of RITUXAN® and B7.1 antibody is well tolerated, the infusion rates of subsequent courses start at 50 mg/hour and escalate at 30 minute intervals by 100 mg/hour increments to a maximum rate not to exceed 300 mg/hr. Vital signs (blood pressure, pulse, respiration, temperature) are monitored every 15 minutes x 4 or until stable, and then hourly until the infusion is completed.

Overall response rate is determined based upon a platelet count determined on two consecutive occasions two weeks apart following the end of the four weekly treatments of RITUXAN® and the three week administration of the B7 antibody composition. Patients treated with the anti-B7.1 antibody and RITUXAN® will show improved platelet counts compared to patients treated with placebo.

While the invention has been described in terms of examples and preferred embodiments, various modifications of the invention in addition to those shown in the art from the foregoing description are included within the scope of the invention. Such modifications are intended to fall within the scope of the following claims.

What is claimed is:

1. Use of an immunoregulatory antibody and an antibody having a B cell depleting activity for the manufacture of a combined preparation for simultaneous separate or sequential use in treating an autoimmune disease in a mammal wherein the immunoregulatory antibody is selected from anti-CD80(B7.1), anti-CD86(B7.2), anti-CD40, and anti-CD4 antibody.

2. Use according to claim 1 wherein the B cell depleting antibody is an antibody that binds an antigen selected from the group consisting of CD10, CD19, CD20, CD21, CD22, CD23, CD24, CD37, CD53, CD72, CD73, CD74, CDw75, CDw76, CD77, CDw78, CD79a, CD79b, CD80 (B7.1), CD81, CD82, CD83, CDw84, CD85 and CD86 (B7.2).

3. Use according to claim 1 or claim 2, wherein the immunoregulatory antibody is an anti-CD80 (B7.1) or anti-CD86 (B7.2) antibody.

4. Use according to any preceding claim, wherein the combination comprises an antibody that binds CD80 (B7.1)and an antibody that binds CD20, CD22, CD19, CD23 or CD37.

5. Use according to any one of claims 1 to 3 wherein the combination comprises an antibody that binds CD86 (B7.2), and an antibody that binds CD19, CD20, CD22, CD23, or CD37.

6. Use according to any preceding claim wherein the immunoregulatory antibody is suitable for administration before the B cell depleting antibody.

7. Use according to any one of claims 1 to 5, wherein the B cell depleting antibody is suitable for administration prior to the immunoregulatory antibody.

8. Use according to any one of claims 1 to 5, wherein the B cell depleting antibody and the immunoregulatory antibody are suitable for administration in combination, essentially at the same time.

9. Use according to any preceding claim, wherein the autoimmune disease is selected from the group consisting of psoriasis; dermatitis; systemic scleroderma and sclerosis; responses associated with inflammatory bowel disease (IBD); Crohn's disease; ulcerative colitis; respiratory distress syndrome; adult respiratory distress syndrome (ARDS); dermatitis; meningitis; encephalitis; uveitis; colitis; glomerulonephritis; allergic conditions; eczema; asthma; conditions involving infiltration of T cells and chronic

inflammatory responses; atherosclerosis; leukocyte adhesion deficiency; rheumatoid arthritis; systemic lupus erythematosus (SLE); diabetes mellitus; multiple sclerosis; Reynaud's syndrome; autoimmune thyroiditis; allergic encephalomyelitis; Sjogren's disease; juvenile onset diabetes; immune responses associated with acute and delayed hypersensitivity mediated by cytokines and T-lymphocytes; tuberculosis; sarcoidosis; polymyositis; granulomatosis; vasculitis; pernicious anemia (Addison's disease); diseases involving leukocyte diapedesis; central nervous system (CNS) inflammatory disorder; multiple organ injury syndrome; hemolytic anemia; myasthenia gravis; antigen-antibody complex mediated diseases; anti-glomerular basement membrane disease; antiphospholipid syndrome; allergic neuritis; Graves' disease; Lambert-Eaton myasthenic syndrome; pemphigoid bullons; pemphigus; autoimmune polyendocrinopathies; Reiter's disease; stiff-man syndrome; Behcet disease; giant cell arteritis; immune complex nephritis; IgA nephropathy; IgM polyneuropathies; idiopathic thrombocytopenic purpura (ITP) and autoimmune thrombocytopenia, and oophoritis.

10. Use according to any preceding claim wherein the mammal is human.

11. Use according to claim 3 wherein neither antibody is conjugated with a cytotoxic agent.

12. Use according to any preceding claim, wherein the antibody combination comprises a humanized or human anti-human CD86 (B7.1) antibody and a chimeric, humanized or human anti-CD20 antibody.

13. Use according to any one of claims 1-10 or 12, wherein the B cell depleting antibody is conjugated with a cytotoxic agent.

14. Use according to claim 13 wherein the cytotoxic agent is a radionuclide.

15. Use according to claim 13 or 14 wherein the antibody comprises Y2B8 or 131I-B1 (BEXXAR™).

16. Use according to any preceding claim wherein the antibodies aresuitable for intravenous administration.

17. Use according to any one of claims 1-15, wherein the antibodies are suitable for administration by infusion.

18. Use according to any preceding claim, wherein a dose of substantially less than 375mg/m^2 of the antibody is suitable for administration to the mammal.

19. Use according to claim 18, wherein the dose is in the range from about 20mg/m^2 to about 250mg/m^2.

20. Use according to claim 19 wherein the dose is in the range from about 50mg/m^2 to about 200mg/m^2.

21. Use according to any preceding claim, wherein an initial dose of the antibody is suitable for administration, followed by a subsequent dose, wherein the mg/m^2 dose of the antibody in the subsequent dose exceeds the mg/m^2 dose of the antibody in the initial dose.

22. Use according to any preceding claim, wherein the autoimmune disease is immune thrombocytopenic purpura (ITP).

23. Use according to any one of claims 1 to 21, wherein the autoimmune disease is rheumatoid arthritis.

24. Use according to any one of claims 1 to 21, wherein the autoimmune disease is hemolytic anemia.

25. Use according to claim 24, wherein the hemolytic anemia is cryoglobinemia or Coombs positive anemia.

26. Use according to any one of claims 1 to 21, wherein the autoimmune disease is vasculitis.

27. Use according to any preceding claim, wherein an anti- CD80 (B7.1) antibody and a B cell depleting anti-CD20 antibody are suitable for administration.

28. An article of manufacture comprising a container and one or several compositions contained therein, wherein at least one composition comprises a B cell depleting antibody, and at least another composition comprises an anti-CD80 (B7.1) or anti-CD86 (B7.2) antibody and further comprising a package insert instructing the user of the composition to treat a patient having or predisposed to an autoimmune disease.

29. The article of manufacture of claim 28 wherein the autoimmune disease is selected from the group consisting of psoriasis; dermatitis; systemic scleroderma and sclerosis; responses associated with inflammatory bowel disease; Crohn's disease; ulcerative colitis; respiratory distress syndrome; adult respiratory distress syndrome (ARDS); dermatitis; meningitis; encephalitis; uveitis; colitis; glomerulonephritis; allergic conditions; eczema; asthma; conditions involving infiltration of T cells and chronic inflammatory responses; atherosclerosis; leukocyte adhesion deficiency; rheumatoid arthritis; systemic lupus erythematosus

(SLE); diabetes mellitus; multiple sclerosis; Reynaud's syndrome; autoimmune thyroiditis; allergic encephalomyelitis; Sjorgen's syndrome; juvenile onset diabetes; immune responses associated with acute and delayed hypersensitivity mediated by cytokines and T-lymphocytes; tuberculosis; sarcoidosis; polymyositis; granulomatosis; vasculitis; pernicious anemia (Addison's disease); diseases involving leukocyte diapedesis; central nervous system (CNS) inflammatory disorder; multiple organ injury syndrome; hemolytic anemia; myasthenia gravis; antigen-antibody complex mediated diseases; anti-glomerular basement membrane disease; antiphospholipid syndrome; allergic neuritis; Graves' disease; Lambert-Eaton myasthenic syndrome; pemphigoid bullous; pemphigus; autoimmune polyendocrinopathies; Reiter's disease; stiff-man syndrome; Behcet disease; giant cell arteritis; immune complex nephritis; IgA nephropathy; IgM polyneuropathies; idiopathic thrombocytopenic purpura (ITP), autoimmune thrombocytopenia and oophoritis.

30. Use of an antibody to CD80 (B7.1) or CD86 (B7.2) and an anti-CD20 antibody having substantial B cell depleting activity, for the manufacture of a combined preparation for simultaneous, separate or sequential use in treating a disease selected from the group consisting of multiple sclerosis, ITP, lupus, diabetes, rheumatoid arthritis, psoriasis, thyroiditis, dermatitis, and IBD.

31. Use according to any one of claims 1 to 27, wherein a synthetic immunosuppresant drug is suitable for administration.

32. Use according to claim 31, wherein said immunosuppressant is cyclosporin or FK506.

33. Use according to claim 31 or claim 32, wherein antibodies targeted against autoantibodies are suitable for administration.